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Antiviral Activity of Favipiravir (T-705) against a Broad Range of Paramyxoviruses *In Vitro* and against Human Metapneumovirus in Hamsters

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The clinical impact of infections with respiratory viruses belonging to the family *Paramyxoviridae* argues for the development of antiviral therapies with broad-spectrum activity. Favipiravir (T-705) has demonstrated potent antiviral activity against multiple RNA virus families and is presently in clinical evaluation for the treatment of influenza. Here we demonstrate *in vitro* activity of T-705 against the paramyxoviruses human metapneumovirus (HMPV), respiratory syncytial virus, human parainfluenza virus, measles virus, Newcastle disease virus, and avian metapneumovirus. In addition, we demonstrate activity against HMPV in hamsters. T-705 treatment inhibited replication of all paramyxoviruses tested *in vitro*, with 90% effective concentration (EC_{90}) values of 8 to 40 μM. Treatment of HMPV-challenged hamsters with T-705 at 200 mg/kg of body weight/day resulted in 100% protection from infection of the lungs. In all treated and challenged animals, viral RNA remained detectable in the respiratory tract. The observation that T-705 treatment had a significant effect on infectious viral titers, with a limited effect on viral genome titers, is in agreement with its proposed mode of action of viral mutagenesis. However, next-generation sequencing of viral genomes isolated from treated and challenged hamsters did not reveal (hyper)mutation. Polymerase activity assays revealed a specific effect of T-705 on the activity of the HMPV polymerase. With the reported antiviral activity of T-705 against a broad range of RNA virus families, this small molecule is a promising broad-range antiviral drug candidate for limiting the viral burden of paramyxoviruses and for evaluation for treatment of infections with (re)emerging viruses, such as the henipaviruses.

The family *Paramyxoviridae* contains a number of viruses causing respiratory tract illnesses in humans, which together have a large clinical impact (1). Human metapneumovirus (HMPV), respiratory syncytial virus (RSV), and parainfluenza virus (PIV) infections are responsible for severe acute respiratory illnesses mainly in young children, but also in immunocompromised and elderly individuals, and are—together with the influenza viruses (family *Orthomyxoviridae*)—the primary viral causes of hospitalizations for severe respiratory tract disease (2–5). No licensed vaccines or effective antiviral treatments are available for these viruses. Measles virus, yet another paramyxovirus, is responsible for a devastating disease which the WHO committed to eradicate with the aid of effective vaccines. However, due to suboptimal immunization levels, resurgences in infections have been detected, and there is no effective antiviral treatment available for measles virus-infected patients (6–8). Paramyxoviruses are well known for their zoonotic potential: avian pneumovirus (AMPV) is the proposed avian ancestor of HMPV, and the avian Newcastle disease virus (NDV) can cause disease—primarily conjunctivitis—in humans (9–11). Multiple novel paramyxoviruses have been detected in bats, including close relatives of human viruses (12, 13), and henipaviruses continue to cause infections in humans in Australia and Asia (14–17). No licensed vaccines or effective antiviral treatments are available for any of these viruses. The continuous burden of disease associated with human and zoonotic paramyxoviruses argues for the development of antiviral therapies with broad-spectrum activity against all paramyxoviruses.

Favipiravir (T-705; 6-fluoro-3-hydroxy-2-pyrazinecarboxamine) is a pyrazine derivative that has demonstrated potent antiviral activity against multiple RNA viruses (18, 19). Intracellular host enzymes act upon T-705, converting it to its active form, T-705-4-ribofuranosyl-5-triphosphate (T-705RTP) (20). T-705RTP functions as a purine nucleotide analog that selectively inhibits the RNA-dependent RNA polymerase (RdRp) or causes lethal mutagenesis upon incorporation into the viral RNA (21–24). T-705 is presently in clinical development as an influenza virus inhibitor in Japan (new drug application filed) and the United States (phase 3 clinical trial) (18). Antiviral activity has been demonstrated against a broad range of negative-strand RNA viruses, such as members of the *Picorna-*, *Arena-*, *Bunya-*, and *Filoviridae* (25–30), and positive-strand RNA viruses, such as the *Noro-* and *Flavivirus* genera (31, 32). Here we evaluated the activity of T-705 against a broad range of paramyxoviruses *in vitro* and against HMPV in hamsters.

MATERIALS AND METHODS

Cells and viruses. The construction of recombinant HMPV (rHMPV) strains NL/00/01-GFP and NL/99/01-GFP and the generation of wild-type HMPV NL/00/17 and NL/94/01, PIV-3 (clinical isolate from 2001),

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and avian pneumovirus type C (AMPV-C) virus stocks in Vero-118 cells have been described previously (33–35). Stocks of measles virus strain Edmonston expressing green fluorescent protein (GFP) (MeV-Edm-GFP; a kind gift of P. Duprex) were generated in Vero-Slam cells as described before (36). A recombinant RSV A2 strain that expresses GFP (RSV-GFP) was a kind gift of M. E. Peeples and P. L. Collins (37). RSV-GFP was grown and titrated in HEp-2 cells in Dulbecco's modified Eagle's medium (DMEM; Lonza, Breda, the Netherlands) supplemented with 2% fetal calf serum. Influenza virus stocks (H1N1 A/Netherlands/602/09) were generated in MDCK cells in DMEM as described previously (38), and virus stocks of herpes simplex virus (HSV) strain F (39) were generated in Vero-118 cells in a manner similar to that for HMPV, without addition of trypsin but with addition of 2% fetal calf serum. Virus stocks of NDV were generated in embryonated chicken eggs as described previously (40). For MeV-Edm-GFP, PIV-3, AMPV-C, and HMPV, infected cells and supernatants were harvested and centrifuged for 5 min at 300 × *g*, the cell-free supernatants were subsequently purified on sucrose gradients (30 to 60%), and aliquots were stored at −80°C. The titers of the virus stocks were determined by endpoint titration on Vero-118 (HMPV, AMPV-C, PIV-3, and NDV), HEp-2 (RSV-GFP), MDCK (influenza virus), or Vero-Slam (MeV-Edm-GFP) cells and expressed as 50% tissue culture infective doses ($TCID_{50}$) per milliliter. Virus titrations were read by either fluorescence assays (rHMPV NL/00/01-GFP, rHMPV NL/99/01-GFP, RSV-GFP, and MeV-Edm-GFP), immunostaining (HMPV NL/94/01 and NL/00/17, AMPV-C, NDV, and PIV-3), or standard hemagglutination assays (influenza virus) or by reading cytopathic effects (HSV).

***In vitro* testing.** T-705 was obtained from Boc Sciences and suspended in dimethyl sulfoxide (DMSO). Serial dilutions of T-705 were made in infection medium, with a final DMSO concentration in the cell culture supernatant of <0.1%. Control samples were treated with amounts of DMSO equal to those used in the treated samples. The activity of T-705 was tested against 100 $TCID_{50}$/well of RSV-GFP in HEp-2 cells, MeV-Edm-GFP in Vero-Slam cells, influenza virus in MDCK cells, and NDV, AMPV-C, PIV-3, and HSV in Vero-118 cells and against 300 $TCID_{50}$/well of the four genotypes of HMPV in Vero-118 cells. Inoculations were performed in infection medium as described in "Cells and viruses" and in references herein. On day 5 (HMPV), 4 (RSV-GFP, NDV, AMPV-C, PIV-3, and MeV-Edm-GFP), or 2 (influenza virus and HSV) after inoculation, 100 μl of supernatant was harvested for virus titration, and cells were examined under a microscope. For viruses not expressing GFP, immunostaining was performed before the microscopic examination. Ninety percent effective concentration (EC_{90}) values were calculated based on the dose-response curves as described previously (41).

Immunostaining assays. Immunostaining for HMPV NL/17/00 and NL/94/01, AMPV-C, and NDV was conducted as described previously (34, 40). Similarly, immunostaining for PIV-3 was conducted using the Imagen immunofluorescence test for PIV from Oxoid (Thermo Fisher Scientific, Landsmeer, the Netherlands).

Cytotoxicity assay. Quadruplicates of 2×10^4 cells per well in 96-well plates (Corning) were either mock inoculated or inoculated with a 2-fold serial dilution series of T-705 in infection medium, starting with a concentration of 250 μM. At 72 h postinoculation, cell viability was determined using the CytoTox 96 nonradioactive cytotoxicity assay (Promega, Leiden, the Netherlands) as described previously (40).

Polymerase assays using minigenomes. The construction and use of a minigenome system for HMPV for polymerase assays have been described previously (33, 42). The minigenome system of HMPV NL/1/00 was used to replace the chloramphenicol acetyltransferase (CAT) open reading frame (ORF) with that of firefly luciferase by using standard PCR and cloning assays. As a control, a plasmid in which firefly luciferase expression was driven by the T7 promoter (in-house construct) was used. To test the effect of T-705 on polymerase activity, 5×10^5 293T cells were plated per well of a 6-well plate in DMEM supplemented with 10% fetal calf serum, 100 U ml^{-1} penicillin, 100 U ml^{-1} streptomycin, 2 mM L-glutamine (PSG), 1 mM sodium pyruvate (Life Technologies, Bleiswijk, the Netherlands), and nonessential amino acids. Cells in each well were transfected via the $CaPO_4$ precipitation method (43) with 1 μg of the minigenome cDNA construct for HMPV NL/1/00, 50 ng pSV40-Renilla-Luciferase (Promega), and 0.8 μg pCITE-N, 0.4 μg pCITE-P, 0.4 μg pCITE M2.1, and 0.4 μg pCITE-L (encoding polymerase complex proteins of HMPV). Control cells for quantification of T7 polymerase activity were established by transfection with 50 ng SV40-Renilla-Luciferase, 1 μg pT7-Firefly-Luciferase, and 1.5 μg pAR3126 (expressing T7 RNA polymerase) (44). Transfections were done overnight, and the medium was refreshed the next morning, with subsequent addition of serial dilutions of T-705. Forty-eight hours after transfection, luminescence was measured as described previously (44).

***In vivo* testing.** Serial dilutions of T-705 were prepared fresh daily by mixing the correct amount of compound in filter-sterilized 0.4% sodium carboxymethyl cellulose (CMC) in Milli-Q water. Six 6-week-old Syrian golden hamsters per group were treated orally with T-705 solution at 25, 50, 100, 150, or 200 mg/kg of body weight twice a day for 4 days, starting 24 h before nasal inoculation with 1×10^6 $TCID_{50}$ HMPV strain NL/00/01. Throat swabs were collected on days 3 and 4 after inoculation and lungs and nasal turbinates (NT) were collected 4 days after inoculation for virus titration and real-time reverse transcription-PCR (RT-PCR) assays.

Virus titration and real-time PCR assays. Collected lungs, nasal turbinates, and throat swabs were processed as described before (45). In brief, tissues were homogenized in infection medium by use of a Polytron homogenizer (Kinematica AG), and supernatants were used for virus titration in Vero-118 cells. Titers were calculated (per gram of tissue), with detection limits of $10^{1.6}$ and $10^{1.2}$ $TCID_{50}$ (g tissue)$^{-1}$ for nasal turbinates and lungs, respectively. Viral RNA was isolated using the MagnaPure LC system with a MagnaPure LC Total nucleic acid isolation kit (Roche Diagnostics, Almere, the Netherlands), with an elution volume of 50 μl. HMPV genome copies were quantitated by TaqMan real-time quantitative RT-PCR (qRT-PCR) as described previously (46).

454 sequencing. Viral RNA was extracted from nasal turbinate and lung samples collected from hamsters, converted to cDNA, and amplified by PCR using primers amplifying 3 overlapping fragments around the polymerase protein gene, covering nucleotides (nt) 6748 to 8974, nt 8330 to 10170, and nt 10158 to 12340, as well as the complete nucleoprotein open reading frame (nt 55 to 1240), in total constituting 51% of the viral genome. PCR fragments for each sample were pooled in equal concentrations, and libraries were created for each sample following the manufacturer's instructions. Emulsion PCR and GS Junior 454 sequencing runs were performed according to the manufacturer's instructions (Roche). Sequence reads were sorted by bar code and trimmed 30 nt from the 3′ and 5′ ends to remove primer sequences, and the 3′ ends were further trimmed to improve quality, using a Phred score of 20. Reads were aligned to the reference sequence for HMPV NL/00/01 (accession no. AF371337.2), using CLC Genomics software 4.6.1. The threshold for low-frequency variant detection was set at 2%, with quality values (*Q*) of ≥20.

Animals and ethics regulation. All experiments involving animals were conducted strictly according to European guidelines (EU directive on animal testing 86/609/EEC) and Dutch legislation (Experiments on Animals Act, 1997). The experimental protocol was reviewed and approved by an independent animal experimentation ethical review committee not affiliated with Erasmus MC (DEC consult number EMC2949).

Statistics. Statistical analysis was performed with GraphPad 6.0 (GraphPad Software). Continuous data between groups were compared using analysis of variance (ANOVA). If this test resulted in a *P* value of <0.05, then pairwise Mann-Whitney tests were performed.

RESULTS

Antiviral activity of T-705 against paramyxoviruses *in vitro*. The inhibitory effect of T-705 on infection was evaluated for RSV-GFP, PIV-3, NDV, AMPV-C, MeV-Edm-GFP, and prototype strains of the 4 genotypes of HMPV (rHMPV NL/00/01-GFP [A1], HMPV NL/00/17 [A2], rHMPV NL/99/01-GFP [B1], and

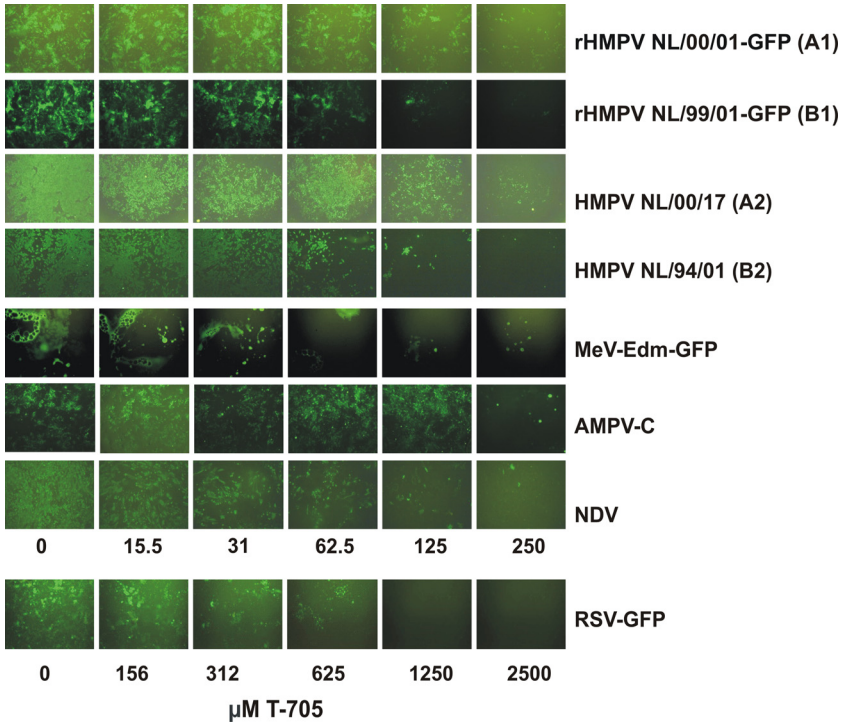

FIG 1 *In vitro* testing for T-705 activity against paramyxoviruses. Cells were treated with serial dilutions of T-705 starting 24 h prior to inoculation with the respective virus. At 5 days postinoculation, GFP-expressing viruses (RSV-GFP, MeV-Edm-GFP, and rHMPV strains NL/00/01-GFP and NL/99/01-GFP) were examined directly under a microscope, and the other viruses were examined after virus-specific immunofluorescence staining as described in Materials and Methods.

NL/94/01 [B2]). The addition of high concentrations of T-705 (up to 1,600 μM) 24 h prior to inoculation had no or little effect on cell viability as measured by cytotoxicity assay. Microscopic examination of the cells revealed that with the addition of increasing T-705 concentrations, the number of infected cells decreased for all viruses tested (Fig. 1). For MeV-Edm-GFP, addition of 62.5 μM T-705 resulted in the absence of typical MeV-Edm-GFP-induced plaques. For most of the other viruses, a decrease in infection efficiency was observed starting at 125 μM T-705. Only for RSV-GFP were higher concentrations of the compound necessary to decrease infection of HEp-2 cells (Fig. 1).

In the next experiment, as a preparation for the *in vivo* experiments, we set out to mimic the effect of T-705 in preexposure and postexposure settings of virus infection *in vitro*. Cells were treated at 3 time points: 24 h prior to, simultaneous with, and 24 h after inoculation. Influenza virus and HSV were used as sensitive and insensitive controls, respectively. Furuta et al. (19) reported HSV to be insensitive to T-705 treatment, with EC_{50} values of >625 μM. In our study, we observed some virus yield reduction when T-705 was administered 24 h prior to and simultaneously with infection, with EC_{90} values of 230 and 540 μM, respectively (Table 1). As these values are significantly higher than those obtained for the sensitive influenza virus ($P = 0.0286$ for both treatment prior to and treatment simultaneous with inoculation; for the Mann-Whitney test, $P < 0.05$) and treatment after infection with HSV had no inhibitory effect on virus release (EC_{90} of >1,500 μM), our data confirm the insensitivity of HSV to T-705 treatment (Fig. 2). Using plaque reduction assays, Furuta et al. reported EC_{50} values for influenza virus ranging from 0.083 to 2.9 μM (19). In the present study, we obtained EC_{90} values of 1.5 μM and 2.0 μM for treatment prior to and simultaneous with infection, respectively. Taking into account that the employed assays and data were different, i.e., plaque reduction assays versus yield reduction assays and EC_{50} versus EC_{90}, these values are in similar micromolar

TABLE 1 *In vitro* activity of T-705 against paramyxoviruses as well as HSV and influenza virus, as controls[a]

Virus	Strain	Prior to inoculation		Simultaneous with inoculation		After inoculation	
		EC_{90} (μM)	SD	EC_{90} (μM)	SD	EC_{90} (μM)	SD
Influenza virus		1.5	1.3	2	2.2	57	35
MeV-Edm-GFP		9	2	10	3	13	7
HMPV A1	NL/00/01	18	11	26	5	27	9
HMPV A2	NL/00/17	26	6	34	7	43	9
HMPV B1	Nl/99/01	13	7	15	10	26	11
HMPV B2	NL/94/01	11	7	12	7	22	13
RSV-GFP		36	12	46	14	69	33
PIV-3		36	3	36	9	68	35
AMPV-C		40	9	50	7	103	35
NDV		48	15	55	6	76	11
HSV		233	39	538	257	>1,500	ND

[a] Cells were treated starting 24 h prior to, simultaneously with, or 24 h after inoculation. Two to 5 days after inoculation, infectious virus yields in the supernatants were determined and EC_{90} values calculated. Different batches of T-705 were tested, and averages and SD are given for two independent experiments, both conducted in duplicate.

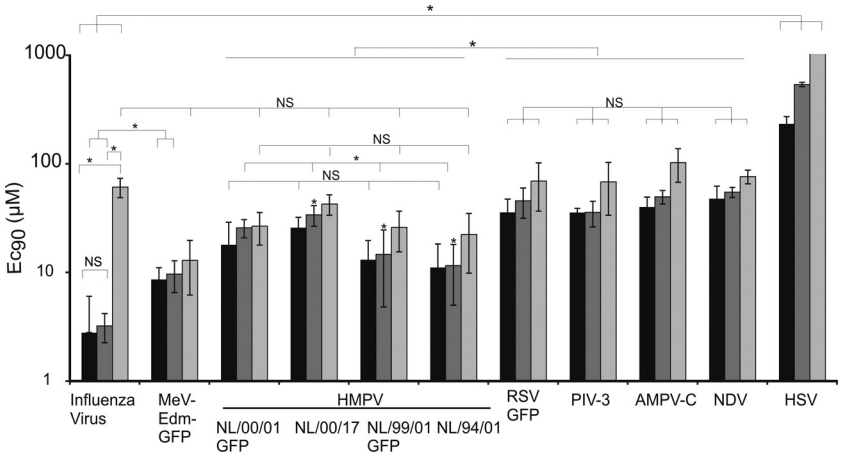

FIG 2 Virus yield reduction assays. *In vitro* activities of T-705 are shown for paramyxoviruses as well as HSV and influenza virus, as controls. Cells were treated starting 24 h prior to inoculation (black bars), simultaneously with inoculation (dark gray bars), or 24 h after inoculation (light gray bars). Two to 5 days after inoculation, infectious virus yields in the supernatants were determined and EC_{90} values calculated. Different batches of T-705 were tested, and averages and ranges are given for two independent experiments, both conducted in duplicate. Statistical analyses were done with one-way ANOVA and Mann-Whitney tests. NS, not significant; *, $P < 0.05$ (ANOVA or Mann-Whitney test).

ranges, which confirms the activity of our batch of T-705 against influenza virus. However, influenza virus was significantly less sensitive to treatment after infection than to treatment prior to or simultaneous with infection ($P = 0.03$ in both cases; for the Mann-Whitney test, $P < 0.05$) (Table 1; Fig. 2).

Of all the paramyxoviruses tested, MeV-Edm-GFP was the most sensitive, with EC_{90} values of 8.6, 9.7, and 13 μM for treatment prior to, simultaneous with, and after inoculation, respectively (Table 1). For treatment prior to or simultaneous with infection, the values are significantly higher than those for influenza virus ($P = 0.03$ in both cases; for the Mann-Whitney test, $P < 0.05$), although the EC_{90} values for MeV-Edm-GFP are still in the micromolar range. MeV-Edm-GFP and influenza virus were similarly sensitive to T-705 treatment 24 h after infection, with EC_{90} values of 57 and 13 μM for influenza virus and MeV-Edm-GFP, respectively ($P = 0.2$; for the Mann-Whitney test, $P < 0.05$). Together, these data show that MeV-Edm-GFP is sensitive to treatment with T-705 over a micromolar range similar to that effective against influenza virus (Fig. 2).

The four genotypes of HMPV were sensitive to treatment in the same micromolar range as that for MeV-Edm-GFP, with EC_{90} values ranging from 11 to 26 μM, 12 to 34 μM, and 22 to 43 μM for treatment prior to, simultaneous with, and after infection, respectively (Table 1). In general, serotype A strains were slightly less sensitive to T-705 than serotype B strains. Although no significant differences were observed between the four strains for treatment before infection ($P = 0.09$; for ANOVA, $P < 0.05$) or after infection ($P = 0.07$; for ANOVA, $P < 0.05$), significant differences between the four HMPVs were observed for treatment simultaneous with infection ($P = 0.003$; for ANOVA, $P < 0.05$) (Fig. 2). This was mainly due to the significantly higher EC_{90} value of NL/00/17 (34 μM) than those of the type B viruses (15 and 12 μM for NL/99/01 and NL/94/01, respectively) for this time of treatment ($P = 0.0286$ in both cases; for the Mann-Whitney test, $P < 0.05$). HMPV strains NL/00/01, NL/99/01, and NL/94/01 were as sensitive as MeV-Edm-GFP to treatment prior to inoculation ($P = 0.34$, 0.48, and 0.52, respectively; for the Mann-Whitney test, $P < 0.05$). Only NL/00/17 was less sensitive than MeV-Edm-GFP to this treatment ($P = 0.03$; for the Mann-Whitney test, $P < 0.05$). All four HMPVs, as well as MeV-Edm-GFP, were as sensitive to treatment after infection as influenza virus ($P = 0.14$; for ANOVA, $P < 0.05$) (Fig. 2). These results obtained for four genotypes of HMPV demonstrate that this virus is sensitive to treatment over a micromolar range similar to that effective against influenza virus and MeV-Edm-GFP.

T-705 also demonstrated an inhibitory effect on virus release for APMV-C, NDV, PIV-3, and RSV (Table 1). No significant differences were observed between these four viruses for the three treatments ($P = 0.35$, 0.08, and 0.37 for treatment prior to, simultaneous with, and after infection, respectively; for ANOVA, $P < 0.05$). However, the EC_{90} values for these four viruses were all significantly higher than those for HMPV strains NL/00/01 ($P = 0.01$, 0.002, and 0.02 for treatment prior to, simultaneous with, and after infection, respectively; for ANOVA, $P < 0.05$), NL/99/01 ($P = 0.002$, 0.0003, and 0.02, respectively), and NL/94/01 ($P = 0.0015$, <0.0001, and 0.01, respectively) but not for HMPV strain NL/00/17 ($P = 0.07$, 0.02, and 0.08, respectively).

Together, these data show that T-705 has an antiviral effect, in the micromolar range, against all the paramyxoviruses tested, with higher EC_{90} values for AMPV-C, NDV, PIV-3, and RSV than for HMPV and MeV-Edm-GFP.

***In vivo* antiviral activity of T-705 against HMPV.** To test the *in vivo* activity of T-705, an established animal model for HMPV infections was employed. Syrian golden hamsters were treated with 50, 100, 150, 200, or 400 mg/kg/day for 4 days, starting 24 h before nasal inoculation with 10^6 $TCID_{50}$ HMPV strain NL/00/01 (type A1). During this experiment, the animals did not show any signs of illness or weight loss.

Real-time RT-PCR assays conducted on throat swabs collected on days 3 and 4 after inoculation and qRT-PCR assays conducted on nasal turbinate and lung samples collected on day 4 after inoculation revealed the presence of viral genomes in these samples at all given concentrations (Fig. 3A and B, top panels). However, the numbers of viral genomes in the throat swabs and nasal turbinates decreased significantly with administration of increasing concentrations of T-705: starting at day 3 for the dose of 100 mg/kg/day

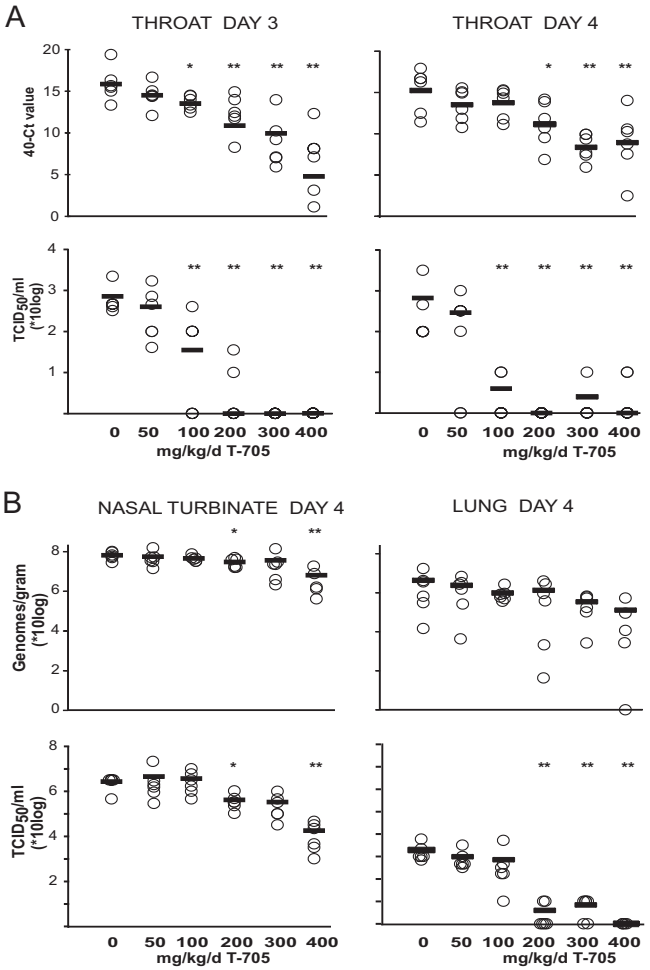

FIG 3 *In vivo* activity of T-705 against HMPV. Six 6-week-old hamsters per group were orally treated with T-705 at 50, 100, 200, 300, or 400 mg/kg/day for 4 days, starting 24 h prior to nasal inoculation with 10^6 $TCID_{50}$ HMPV strain NL/1/00. Throat swabs were collected on days 3 and 4, and nasal turbinates and lungs were collected at 4 days postinoculation. (A) C_T values obtained by real-time RT-PCR assays (top panels) and viral titers (bottom panels) for throat swabs collected on day 3 (left panels) and day 4 (right panels). (B) Numbers of HMPV genomes as determined by qRT-PCR (top panels) and viral titers (bottom panels) per gram of nasal turbinate (left panels) or lung (right panels). Circles indicate values for individual animals, and black lines show average values for 6 animals. *, $P < 0.05$; **, $P < 0.01$ (Mann-Whitney test).

($P = 0.026$; for the Mann-Whitney test, $P < 0.05$) and at day 4 for the dose of 200 mg/kg/day ($P = 0.01$; for the Mann-Whitney test, $P < 0.05$) for the throat swabs and at a dose of 200 mg/kg/day ($P = 0.026$; for the Mann-Whitney, $P < 0.05$) for the nasal turbinates. T-705 treatment did not result in a significant decrease in viral genome titers in the lungs ($P = 0.2$; for ANOVA, $P < 0.05$), although the titers did decrease with increasing concentrations of T-705 (Fig. 3B). Virus titration of the throat swab samples revealed levels of infectious virus below the limit of detection in 3 (day 3) or 4 (day 4) of the 6 animals treated at a dose of 100 mg/kg/day. In general, infectious virus titers decreased significantly in the throat swabs starting at a dose of 100 mg/kg/day ($P = 0.0022$; for the Mann-Whitney test, $P < 0.05$) (Fig. 3A, lower panels) and in the nasal turbinates and lungs at 200 mg/kg/day ($P = 0.015$ and $P = 0.022$ for nasal turbinates and lungs, respectively; for the Mann-Whitney test, $P < 0.05$) (Fig. 3B, lower panels). Most importantly, at a dose of 200 mg/kg/day, 3 of 6 animals displayed levels of infectious virus in the lungs below the limit of detection, and at a dose of 400 mg/kg/day, none of the animals had levels of infectious virus in the lungs above the limit of detection (Fig. 3B, lower right panel).

Although replicating virus could not be detected in the lungs of animals treated with a dose of 400 mg/kg/day, viral genome titers declined only minimally. These findings of decreased infectious virus titers in samples from treated animals and only limited decreases in viral genome titers resemble the findings reported for influenza virus. In the influenza virus study, the infectious virus load in treated samples decreased disproportionately to the RNA copy number. It was demonstrated that T-705 induced hypermutation of the viral genome, which explained the decrease in viral titers with equal titers of viral genome copies in samples treated with T-705 (24). To investigate this possibility for the mechanism of action of T-705 against HMPV, samples (nasal turbinates and lungs) from untreated animals and high-dose-treated animals were subjected to next-generation sequencing (Table 2). To this end, the region from nt 6748 to 12340 of the viral genome (the L open reading frame), for two animals each (untreated animals H013 and H014 and treated animals H044 and H045), was subjected to 454 deep sequencing using 3 overlapping PCR fragments. Overall, this region was covered by 6,892 to 17,447 reads (Fig. 4A), and analysis of single nucleotide polymorphisms (SNPs) revealed the presence of 22 SNPs in the viral genomes isolated from treated animals that were also present in the viral genomes retrieved from untreated hamsters (see Table S1 in the supplemental material). This analysis also revealed the presence of approximately 25 reads with T-to-C hypermutation, but these were detected only for the sample isolated from the nasal turbinate of one treated hamster (animal H043) (Fig. 4A, blue circles around nt 8000). These hypermutated reads were absent for the lung sample from the same hamster and the samples from the other treated hamster (H044). These hypermutated reads most likely represent (hypermutated) defective interfering virus particles (DIs) that occur during HMPV infection, as previously reported (44), and based on their absence in the other (treated) samples, they were not a result of T-705 treatment. The T-C hypermutation detected in these reads was included in the calculation of the number of SNPs for Fig. 4C, which explains the wide error bars.

Additional SNPs were detected that were present in untreated samples and absent in treated samples or the other way around. In the nasal turbinate samples from untreated animals, 9 (H013) and

TABLE 2 Information on treatment, C_T values obtained by qRT-PCR, and virus titers for hamster samples selected for deep sequencing

Animal	T-705 dose (mg/kg/day)	Lungs		Nasal turbinates	
		C_T	Titer ($TCID_{50}$/g)	C_T	Titer (no. of genomes/g)
H013	0	18	7.2×10^3	17	9.39E+07
H014	0	25	3.4×10^3	19	4.60E+07
H015	0	21	3.4×10^3	19	2.60E+07
H041	300	25	0	20	2.09E+07
H044	400	23	0	21	1.87E+07
H045	400	24	0	21	7.25E+06

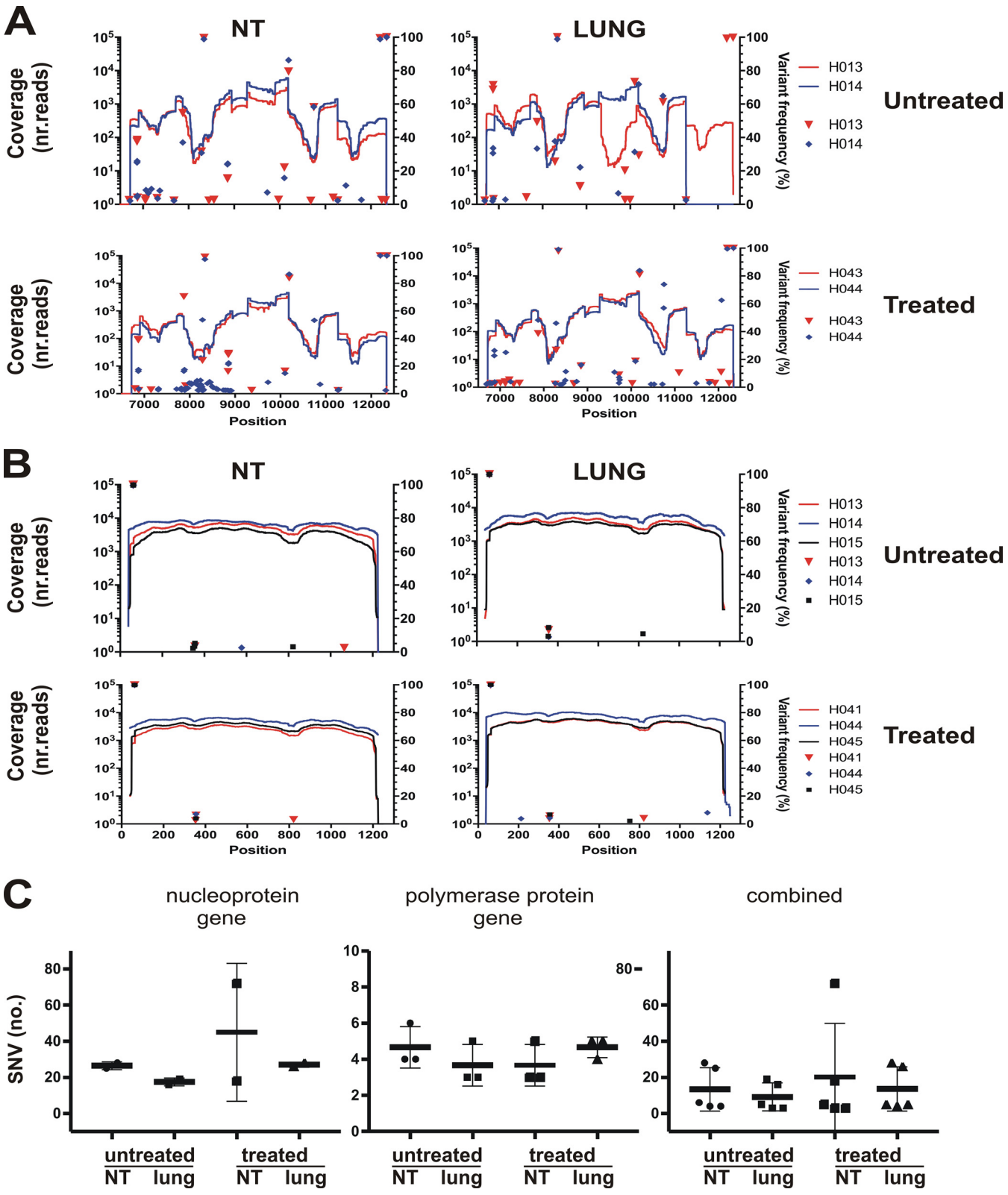

FIG 4 Detection of mutations in the polymerase protein gene (A) and the nucleoprotein gene (B) of HMPV by 454 sequencing. Graphic representations are shown for coverage and viral variant analysis of nasal turbinates (NT) and lung tissue for untreated hamsters (H013, H014, and H15) and hamsters treated with T-705 at 300 mg/kg/day (H041) or 400 mg/kg/day (H044 and H045). Lines indicate the coverage over the target region (left *y* axis). Blue diamonds, black dots, and red triangles indicate variants detected for each sample. Positions of dots on the *x* axis indicate the positions in the sequence where variations were found. Positions on the right *y* axis indicate the amounts of variation at those positions. (C) Numbers of SNPs detected in the polymerase protein gene region, the nucleoprotein gene region, and both regions combined for viral genomes isolated from nasal turbinates (NT) and lungs from treated and untreated animals. Black spots indicate individual SNP numbers for animals, the black (horizontal) lines show the averages for all animals, and standard deviations are indicated. Using the Mann-Whitney test ($P < 0.05$), no significant differences in numbers of SNPs between untreated and treated animals were found.

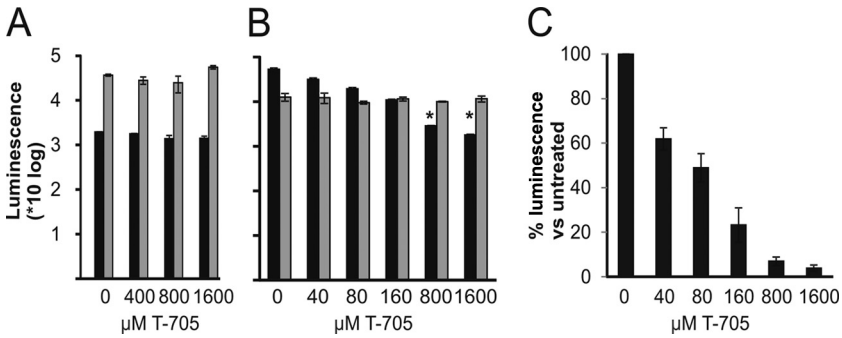

FIG 5 Effect of T-705 on polymerase activity. 293T cells transfected with T7-Firefly-Luciferase and a transiently expressed control (SV40-Renilla-Luciferase) (A) or the HMPV minigenome system expressing firefly luciferase and the transiently expressed control (SV40-Renilla-Luciferase) (B) were treated with serial dilutions of T-705 18 h after transfection. Luminescence was read 48 h after transfection as described in Materials and Methods. Black bars, firefly luciferase activity; gray bars, renilla luciferase activity. (C) Percentages of luminescence compared to that of the untreated samples (set to 100%). Graphs represent values obtained in one representative experiment conducted in duplicate. *, $P < 0.05$ (*t* test).

10 (H014) SNPs were detected that were absent in samples from treated animals, and in the lung samples, 4 (H013) and 5 (H014) SNPs were detected that were absent in samples from treated animals; all of these were present in <10% of the reads (see Table S1 in the supplemental material). In the nasal turbinate samples from treated animals, 4 SNPs (in both H043 and H044) were detected that were absent in the untreated samples, and in the lung samples, 16 (H043) and 4 (H044) SNPs were detected that were absent in the untreated samples. Overall, the numbers of SNPs detected in this part of the genome did not differ significantly between the treated and untreated samples, even when the 25 hypermutated reads detected in the nasal turbinate sample from animal H043 were included (Fig. 4C). To obtain more evidence and to avoid the detection of DIs, the nucleoprotein gene of the genomes isolated from the same animals, and including a third animal per group (H015 or H041), was also subjected to deep sequencing. To this end, one PCR fragment covering the complete ORF was analyzed, and deep sequencing resulted in coverage of this region by 22,655 to 49,335 reads (Fig. 4B). Similar to the results obtained for the polymerase protein ORF, SNPs detected in the viral genomes isolated from treated animals were also present in those obtained from untreated animals and were present in fewer than 10% of the reads (see Table S2 in the supplemental material). There were no significant differences between the numbers of SNPs detected in the nucleoprotein ORF for the treated and untreated samples (Fig. 4C). Thus, next-generation sequencing of approximately 50% of the viral genome in samples obtained from treated and untreated animals did not reveal significant differences in SNPs detected in treated and untreated samples (Fig. 4C), which indicates that T-705 did not induce specific mutations in the analyzed parts of the viral genome.

To investigate whether T-705 has a direct effect on the activity of the polymerase proteins of HMPV, as described for those of influenza virus and chikungunya virus (18, 47), the inhibitory effect of T-705 on polymerase complex activity was evaluated by use of a minigenome system. Addition of DMSO alone (data not shown) or increasing concentrations of T-705 had no inhibitory effect on the expression of the constitutively expressed renilla luciferase or on the expression of a T7 promoter-driven firefly luciferase expression plasmid (Fig. 5A). In contrast, addition of increasing concentrations of T-705 to the minigenome system for HMPV, expressing firefly luciferase, demonstrated an inhibitory effect of T-705 on the HMPV polymerase activity (Fig. 5). Addition of 80 μM T-705 reduced the activity by 50% compared to that in untreated samples, while addition of 800 μM T-705 significantly reduced the luciferase activity, to below 10% ($P = 0.002$; for the *t* test, $P < 0.05$). These data demonstrate that for HMPV, the mechanism of action of T-705 is at least partially directed against the polymerase activity and is not the induction of lethal mutagenesis of viral genomes.

DISCUSSION

T-705 has been shown to be effective against important respiratory viruses, such as influenza viruses and rhinoviruses. In this study, we demonstrated the activity of T-705 against important human respiratory viruses of the family *Paramyxoviridae*: HMPV, RSV, PIV, and measles virus. The EC_{90} values detected *in vitro* were in the low micromolar range (on average, below 10 μg/ml), similar to values observed for influenza virus (EC_{50} values of 0.013 to 0.46 μg/ml) (19), bunyaviruses (5 to 30 μg/ml), arenaviruses (0.7 to 1.2 μg/ml), and picornaviruses (4.8 to 23 μg/ml) (25). Using plaque reduction assays, Furuta et al. (19) reported an EC_{50} of 260 μM for RSV. In our yield reduction assays, we found EC_{90} values of 36 to 70 μM for the different treatment regimens. In both studies, lab-adapted strains of RSV were used. The activity of T-705 against RSV might be higher against wild-type strains, which would be worthwhile to investigate.

In addition, differences between observed values may be related to the use of different assays, such as plaque reduction assays versus virus yield reduction assays, or to presentation of the data as EC_{50} or EC_{90}, but in general, the values are sufficiently low to be translated for *in vivo* use.

Our results also demonstrate activity against the avian paramyxoviruses AMPV-C and NDV. Although these viruses do not cause substantial disease in humans, paramyxoviruses are well known for their zoonotic potential. The activity against these avian viruses reveals that T-705 has activity against a broad range of paramyxoviruses, and therefore it is possible that this compound will also be active against henipaviruses.

To translate *in vitro* activity to *in vivo* activity, we employed an established hamster model of HMPV infection (45). In this model, T-705 treatment decreased virus replication in the throats, nasal turbinates, and lungs of the inoculated animals starting at a dose of 200 mg/kg/day, and at a dose of 400 mg/kg/day, none of the

animals had detectable levels of infectious virus in their lungs. Previous vaccination studies for HMPV have also resulted in protection of the lungs but not the nasal turbinates upon challenge. The primary goal of treatment of respiratory virus infections is prevention of serious lower respiratory tract illnesses, and in this *in vivo* model, treatment with T-705 protected against infection of the lungs. The dose of 200 to 400 mg/kg/day is similar to doses used in other animal models for other viruses (19). In addition, these doses are within the range reported for influenza virus infection, for which the compound is now approved in Japan and is being evaluated in clinical trials elsewhere (www.clinicaltrials.gov).

Our *in vitro* pre- and postexposure experiments revealed that T-705 is more effective when treatment starts 24 h before infection or during infection. Especially for influenza virus and HSV, which are both fast-replicating viruses, late addition of the compound was less effective. Although EC_{90} values were higher on late addition for the paramyxoviruses, values for all three treatments were roughly in the same range.

Because T-705 needs to be metabolized into its active metabolite, T-705-4-ribofuranosyl-5-monophosphate (T-705RMP), and then to T-705-4-ribofuranosyl-5-triphosphate T-705RTP, it requires several hours to reach an effective concentration (21, 48). This may explain why the compound is less effective against fast-replicating viruses when treatment occurs postinfection. Comparison of the results obtained for inhibition of infection (Fig. 2) and for virus yield reduction (Fig. 3) demonstrated that T-705 had a larger effect on virus yield than on virus infection, as lower concentrations of compound were needed to reduce the virus yield than to inhibit virus infection.

The mechanism of action of T-705 remains a topic of investigation. Both direct inhibition of the polymerase and induction of lethal mutagenesis have been described (23, 24, 47, 49). In the T-705-treated hamsters inoculated with HMPV, viral genomes were detected in all animals even when the animals were treated at high doses. This presence did not always correlate with the presence of infectious virus, suggesting an effect of the compound on the formation of infectious virus. This is further supported by our data showing that treatment had a larger effect on virus yield than on viral infection (Fig. 3 versus Fig. 2). Deep sequencing of approximately half of the viral genome for samples obtained from untreated and treated animals did not reveal any T-705-induced mutations in the analyzed parts of the viral genome. As no materials were left for sequencing of other parts of the genome, we cannot rule out the presence of mutations in other parts of the genome that could explain the mechanism of action of T-705 against HMPV. However, the mutations detected in the aforementioned studies on influenza virus, norovirus, and chikungunya virus were induced during passaging of the virus under the pressure of high concentrations of T-705. Whether these types of mutations also occur in *in vivo* situations remains unknown. In a mouse model of persistent norovirus infections where the animals were treated for 8 weeks, an average 2.9-fold increase in mutation frequency was found for virus samples obtained from T-705-treated animals compared to control animals (49). However, in this case, the virus was under pressure for a prolonged period (8 weeks), which cannot be compared to the 4-day treatment in the HMPV hamster model.

We did detect T-C hypermutation in genomes obtained from the nasal turbinate of one treated animal. These were all detected in approximately 25 (hypermutated) reads. This type of read, with T-C hypermutation, has been detected previously during HMPV infections *in vitro* (44) and is therefore most likely not a result of T-705 treatment. This is, as far as we know, the first time that HMPV DIs were detected during *in vivo* infection, which will be interesting for future investigations.

The absence of (hyper)mutations in the nucleoprotein gene, the region where the qRT-PCR primers align, proves that the increase in threshold cycle (C_T) values for T-705-treated samples (Fig. 3) was not due to a changed sensitivity of the qRT-PCR assay but to the activity of T-705.

Using minigenome assays to investigate the effect of T-705 on the polymerase activity of HMPV, we showed that adding T-705 to this assay resulted in a significant decrease in the polymerase activity. At present, there are two hypotheses for the mechanism of action of T-705: (i) the induction of lethal mutagenesis and (ii) chain termination by the incorporation of favipiravir (T-705) into the nascent RNA strand (23). Our data indicate that T-705 does not induce lethal mutagenesis on HMPV genomes but does have a direct effect on the HMPV polymerase. This is in line with the activity against the influenza virus RNA-dependent RNA polymerase reported by Kiso et al. (50) and with data reported for the chikungunya virus, in which a single mutation in the polymerase protein was sufficient to make the virus resistant to treatment (47). Although it is possible that T-705 interferes with viral morphogenesis at a late stage of the infectious cycle, T-705 is a nucleotide analog, and its mechanism of action is thought to be related to the selective inhibition of viral RNA-dependent RNA polymerases of influenza virus and many other RNA viruses (21, 23). Time-of-addition experiments have demonstrated that T-705 inhibits the early to intermediate stage of viral replication of arenaviruses and noroviruses (32, 51). However, the exact mechanism of action against paramyxoviruses still remains to be elucidated.

In conclusion, we show here that T-705 has antiviral activity against a broad panel of paramyxoviruses, both human and avian. With the reported antiviral activity of T-705 against a broad range of RNA virus families, this small molecule is a promising broad-range antiviral drug candidate for limiting the viral burden of respiratory viruses and for evaluation for treatment of (re)emerging viruses, such as the henipaviruses. Importantly, elucidation of the mechanism of action of T-705 against all these viruses would allow the design of novel compounds that employ similar mechanisms of action, which may help in the development of antiviral treatment options for a broad spectrum of respiratory viruses.

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